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Zn-α_2-glycoprotein (ZAG) is a soluble protein that is present in serum and other body fluids. In addition, ZAG accumulates in breast cysts as well as in 40% of breast carcinomas. ZAG stimulates lipid degradation in adipocytes and causes the extensive fat losses associated with some advanced cancers. The 2.8 Å crystal structure of ZAG resembles a class I major histocompatibility complex (MHC) heavy chain, but ZAG does not bind the class I light chain β_2-microglobulin. The ZAG structure includes a large groove analogous to class I MHC peptide binding grooves. Instead of a peptide, the ZAG groove contains a non-peptidic compound that may be implicated in lipid catabolism under normal or pathological conditions.

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Luis Salvato

PI - Signature

24/9/1999

Date

Table of contents

Introduction

Zn-α_2-glycoprotein (ZAG) is a soluble protein related to class I major histocompatibility complex proteins (MHC). ZAG is present in serum and other body fluids; in addition, it accumulates in breast cysts as well as in 40% of breast carcinomas. ZAG stimulates lipid degradation in adipocytes and causes the extensive fat losses associated with some advanced cancers. Our purpose is to determine the three-dimensional structure of ZAG. The information thus obtained will be the necessary basis for understanding the role of this protein in fat metabolism, as well as in breast cancer.

Body

The only strategy capable to determine the structure of a macromolecule of 40 kDa, such as ZAG, is x-ray analysis of crystals of the macromolecule. Therefore, our first task was to obtain crystals of ZAG. The requirements for crystallizing a protein are 3-50 mgs of native protein that is both pure and conformationally homogeneous. We purified ZAG from human serum and eventually obtained crystals of the protein that were suitable for x-ray analysis.

Data obtained by crystallographic x-ray analysis are converted into a list of thousands of intensities. However, these intensities are not enough to solve the structure of macromolecules. Each intensity has to be complemented by an additional value, its "phase", in order to reconstruct the image of the macromolecule. So, our next task was to obtain those phases. We estimated preliminary values for the phases by multiple isomorphous replacement (MIR). MIR is an experimental approach where protein crystals are soaked with different compounds containing heavy atoms. Comparison of x-ray data sets from soaked and non-soaked crystals can produce approximate values for the phases.

The image of the protein obtained by these studies is always blurred due to the inevitable errors in the measurements, particularly in the phases. We obtained this blurred image at the end of the previous research period. During the current research period, we have refined this image and built the atomic structure of ZAG inside it. Therefore, we have finally solved the three-dimensional structure of ZAG [1]. ZAG has a "L" shape in which the long axis of its α_3 domain is roughly perpendicular to the flat side of a platform formed by its α_1 and α_2 domains.

ZAG is the first known structure of a MHC-related protein that does not bind β_2-microglobulin (β_2M). β_2M is necessary for the correct folding of most MHC-related proteins and is tightly bound by the other domains of these

molecules. Later, the structure of another MHC-related protein that does not bind β_2M (MIC-A) was published [2] and shows severe distortions when compared with typical MHC molecules. However, ZAG remains folded in the absence of β_2M, and shows only minor rearrangements of its quaternary structure. Comparison of the interface between the α_1–α_2 platform and the α_3 domain reveals that an expanded hydrogen-bond network and an enlarged interdomain surface stabilize ZAG in the absence of β_2M.

The positions of some of the carbohydrates of ZAG are stabilized by crystal contacts. This fact allows the observation of nine residues in a biantennary structure, forming one of the biggest carbohydrate structures ever observed in a protein.

ZAG contains a large binding groove analogous to class I MHC peptide binding grooves. However, instead of a peptide, the ZAG groove contains a non-peptidic compound. This observation is perhaps the most important conclusion of this work, since it reveals that ZAG is a carrier of hydrophobic ligands that may be implicated in lipid catabolism and in the pathogenesis of breast cancer.

References

1. Sánchez, L.M., A.J. Chirino, and P.J. Bjorkman, *Crystal structure of human ZAG, a fat-depleting factor related to MHC molecules.* Science, 1999. **283**(5409): p. 1914-9.

2. Li, P., *et al.*, *Crystal structure of the MHC class I homolog MIC-A, a gammadelta T cell ligand.* Immunity, 1999. **10**(5): p. 577-84.

Key research accomplishments

1. The structure of ZAG has been determined. I have learned protein crystallography and plan to use these skills to solve the three-dimensional structure of other proteins related to breast cancer.
2. The structure of ZAG reveals that this protein is a carrier of a hydrophobic non-peptidic ligand. I am using these results for identifying the ligand and planning mutagenesis experiments to characterize the function of ZAG in breast cancer and in the regulation of fat metabolism and body weight.
3. Comparison of the structures of ZAG and other members of the MHC family illuminates the role of β_2M in folding and stability, as well as the structural and functional differences between members that act as carriers of low molecular weight compounds and members that perform other functions.

Reportable outcomes

Sánchez, L.M., A.J. Chirino, and P.J. Bjorkman, *Crystal structure of human ZAG, a fat-depleting factor related to MHC molecules.* Science, 1999. **283**(5409): p. 1914-9.

The results of this article have been commented in the Caltech Press Releases (http://www.caltech.edu/~media/Press_Releases/PR11977.html), and in the Howard Hughes Medical Institute News (http://www.hhmi.org/news/bjorkman.htm).

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SCIENCE

Crystal Structure of Human ZAG, a Fat-Depleting Factor Related to MHC Molecules

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Crystal Structure of Human ZAG, a Fat-Depleting Factor Related to MHC Molecules

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Zn-α_2-glycoprotein (ZAG) is a soluble protein that is present in serum and other body fluids. ZAG stimulates lipid degradation in adipocytes and causes the extensive fat losses associated with some advanced cancers. The 2.8 angstrom crystal structure of ZAG resembles a class I major histocompatibility complex (MHC) heavy chain, but ZAG does not bind the class I light chain β_2-microglobulin. The ZAG structure includes a large groove analogous to class I MHC peptide binding grooves. Instead of a peptide, the ZAG groove contains a nonpeptidic compound that may be implicated in lipid catabolism under normal or pathological conditions.

ZAG is a soluble protein whose name derives from its tendency to precipitate with zinc salts and its electrophoretic mobility in the region of the α_2 globulins (*1*). ZAG is normally present in most body fluids including serum, sweat, saliva, cerebrospinal fluid, seminal plasma, milk, amniotic fluid, and urine (*1*). In addition, ZAG accumulates in breast cysts as well as in 40% of breast carcinomas, and is induced by glucocorticoids and androgens in breast cancer cell lines. Hence, ZAG may participate in breast diseases, including cancer (*2*).

The function of ZAG was elucidated when a lipid-catabolizing factor with the same amino acid sequence as ZAG was isolated from the urine of cancer patients with cachexia (*3*). Cachexia is a wasting syndrome caused by depletion of muscle and adipose tissue that is present in the majority of patients with cancer, AIDS, and other life-threatening diseases (*3*). ZAG appears to be responsible for the fat-depletion component of cachexia, since it stimulates lipid breakdown in adipocytes and reduces fat stores in laboratory animals (*3*). ZAG is overexpressed in carcinomas that induce fat loss but not in other tumors. Application of ZAG to adipocyte membranes activates a guanosine triphosphate–dependent adenylate cyclase activity, perhaps through direct or indirect interactions with a G protein–coupled receptor (*3*). Thus, its mode of action could be similar to that of lipolytic hormones. These results suggest that ZAG normally functions to regulate lipid degradation, which increases to a pathological extent in cachexia.

ZAG shares 30 to 40% amino acid sequence identity with the extracellular portions of class I major histocompatibility complex (MHC) heavy chains (*4*). Class I MHC molecules present peptide antigens to cytotoxic T cells (*5*). Other proteins related to class I MHC molecules include CD1, which presents hydrophobic antigens to T cells (*6*), the neonatal Fc receptor (FcRn), which transports immunoglobulin G across epithelia (*7*), and HFE, which binds transferrin receptor and regulates iron homeostasis (*8*). These MHC homologs are membrane-bound heterodimers that use the soluble protein β_2-microglobulin (β_2M) as a light chain. ZAG, however, is a secreted protein, and it does not associate with β_2M (*9*). The latter property is shared by MIC-A, a divergent membrane-bound member of the class I family (*10*).

Like FcRn (*11*), HFE (*11*), and MIC-A (*10*), ZAG does not bind endogenous peptides (*9*), but it appears to carry a small proteinase-resistant compound whose injection induces glomerulonephritis in experimental animals (*12*). In peptide-binding class I MHC molecules, a large groove located between two α helices in the α1-α2 superdomain of the heavy chain serves as the binding site (*5*). An analogous groove acts as the antigen binding site in CD1,

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but is narrowed in HFE and closed in FcRn, the class I–related proteins that do not bind small molecular weight ligands (*11*).

Here, we present the 2.8 Å crystal structure of human ZAG, which reveals an MHC-like fold without a β_2M light chain and a groove that closely resembles the peptide-binding sites of classical class I MHC molecules. Rather than containing a peptide, the

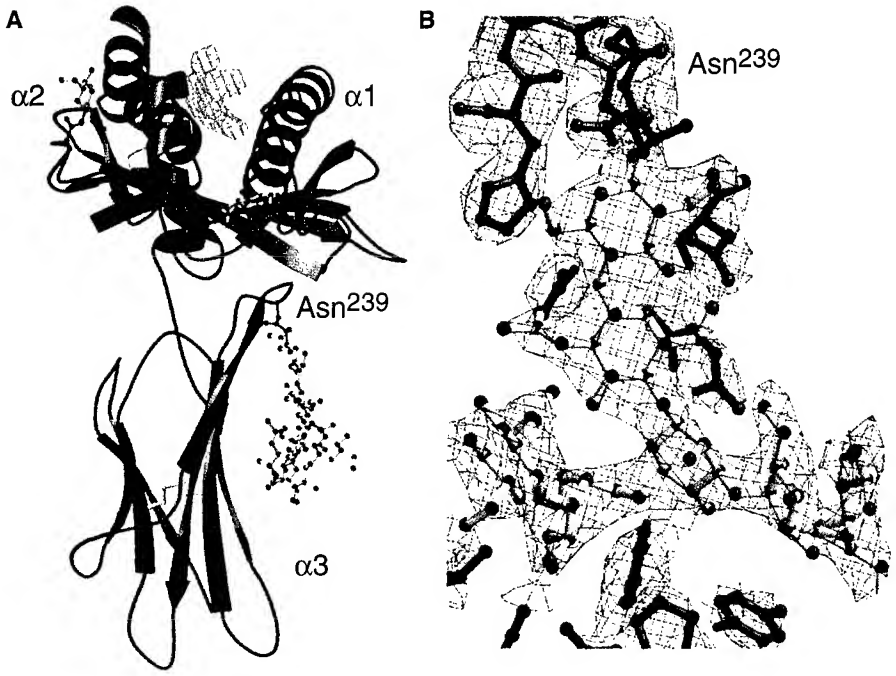

Fig. 1. (**A**) Ribbon diagram of the structure of human ZAG. Density corresponding to the non-peptidic ligand is shown in green as derived from a 2.9 Å MIRAS, NCS averaged, figure-of-merit weighted electron density map contoured at 1σ. Ordered N-linked carbohydrates are shown in ball-and-stick representation. (**B**) The ZAG model (molecule 2) in the region of the N-linked carbohydrate attached to Asn^{239} superimposed on a 2.8 Å SIGMAA-weighted $2F_o - F_c$ annealed omit electron density map (*13*). The carbohydrate electron density at Asn^{239} shows nine carbohydrate residues arranged in a biantennary structure (*14*) (two of the carbohydrate residues are omitted from the figure for clarity). The large number of ordered carbohydrate residues at Asn^{239} is likely to result from stabilization of the flexible carbohydrate by crystal contacts with protein residues in symmetry-related ZAG molecules (shown in red sticks; bottom). Figures were made as described (*23*).

Table 1. Data collection, heavy-atom phasing, and refinement statistics for ZAG. Statistics in parentheses refer to the highest resolution bin. ZAG was crystallized in space group $P2_12_12$ with four molecules per asymmetric unit and cryoprotected as described (*9*). Native and heavy-atom derivative data sets were collected at −165°C from multiple crystals using an R-AXIS IIc imaging plate system mounted on a Rigaku R200 rotating anode generator. Data were processed and scaled with the HKL package (*13*). Heavy-atom refinement was done with SHARP (*13*), which treated different data sets of derivatives with the same sites as separate "crystals" of the same "compound" and refined separate heavy-atom occupancies and temperature factors for each "crystal" data set. Electron density maps calculated from MIRAS phases derived from all eight heavy-atom data sets were superior to maps derived from various combinations of mercury and platinum data sets. Eight NCS operators were determined from least squares superposition of HLA-A2 α1-α2 and α3 domains manually placed in the electron density, four relating the α1-α2 regions and four relating the α3 domains. After solvent flipping using Solomon (*13*), subdomain NCS averaging of the four molecules and phase extension from 5.0 to 2.9 Å were carried out with DM in the CCP4 suite (*13*) (final average correlation >0.8). When the same averaging and phase extension procedure was done without prior solvent flipping, the resulting electron density maps were inferior. The model was built using O (*13*) and refined as described (*13*).

Data set	Resolution (Å)	Complete (%)*	R_{merge}(%)†	I/σI	rms f_h/E‡
Native I	2.9	97.0 (83.0)	10.7 (29.7)	27.9 (3.2)	
Native II	2.8	96.0 (76.0)	6.6 (45.3)	24.7 (2.5)	
Mercury acetate					
1	3.4	94.4 (97.5)	13.6 (30.4)	10.8 (4.4)	3.0
2	4.0	94.4 (97.2)	17.9 (35.5)	6.8 (3.3)	2.2
3	3.4	98.5 (99.1)	13.2 (32.4)	9.9 (3.9)	2.8
4	3.2	97.7 (93.3)	7.8 (30.8)	19.1 (4.3)	1.9
PIP§					
1	3.7	91.8 (77.3)	5.7 (35.4)	22.1 (3.5)	1.1
2	3.2	98.5 (97.7)	7.3 (33.6)	22.0 (4.1)	0.8
3	3.6	95.4 (96.4)	8.1 (29.5)	20.8 (5.2)	1.0
K_2PtCl_4	4.0	98.8 (99.3)	13.0 (39.5)	10.1 (3.1)	1.5

Refinement statistics				
Resolution (Å)		20.0–2.8	rms ΔB bonded NCS atoms (Å^2)#	14.4, 15.4, 19.0
Reflections in working set	$\|F\| > 0$	37148	rms Δφ all NCS residues (degrees)#	2.4, 3.2, 3.7
Reflections in test set	$\|F\| > 0$	1970	rms Δψ all NCS residues (degrees)#	2.0, 2.3, 2.4
R_{free} (%) ‖		28.8		
R_{cryst} (%) ¶		22.9	Number of nonhydrogen atoms	
rms deviations from ideal			Protein	8866
Bond lengths (Å)		0.008	Carbohydrate	388
Bond angles (degrees)		1.35	Nonglycine residues in allowed regions of Ramachandran plot as defined (*13*)	96%

*Complete = (number of independent reflections)/total theoretical number. †R_{merge} (I) = $[\Sigma|I(i) - \langle I(h)\rangle|/\Sigma I(i)]$, where I(i) is the *i*th observation of the intensity of the hkl reflection and ⟨I⟩ is the mean intensity from multiple measurements of the hkl reflection. ‡rms f_h/E (phasing power), where f_h is the heavy-atom structure factor amplitude and *E* is the residual lack of closure error. §PIP, di-m-iodobis(ethylenediamine)diplatinum nitrate. ‖R_{free} is calculated over reflections in a test set not included in atomic refinement. ¶R_{cryst} (*F*) = $\Sigma_h||F_{obs}(h)| - |F_{calc}(h)||/\Sigma_h|F_{obs}(h)|$, where $|F_{obs}(h)|$ and $|F_{calc}(h)|$ are the observed and calculated structure factor amplitudes for the hkl reflection. #Statistics for NCS-related residues refer to differences relative to molecule 1 for the other three molecules in the crystallographic asymmetric unit.

ZAG groove includes an unidentified nonpeptidic ligand that may be relevant for ZAG's function in lipid catabolism.

ZAG was purified from human serum and crystallized as described (*9*). The crystal structure was determined by multiple isomorphous replacement with anomalous scattering (MIRAS) aided by fourfold noncrystallographic symmetry (NCS) averaging (Table 1) (*13*). The overall structure of ZAG is similar to those of class I MHC heavy chains (Fig. 1A). The α1-α2 superdomains of ZAG, class I, and class I–related proteins form a single eight-stranded antiparallel β sheet topped by two α helices, and the α3 domain adopts a fold resembling immunoglobulin constant domains (*5, 11*). Electron density corresponding to carbohydrate is present at three of the four potential N-linked glycosylation sites in ZAG, with an unusually large number of ordered carbohydrate residues visible at Asn^{239} (Fig. 1B) (*14*). The α3 domain of human, but not mouse or rat, ZAG contains an RGD sequence (Arg^{231}, Gly^{232}, Asp^{233}) suggested to be involved in cell adhesion (*15*). However, unlike the RGD sequences in characterized adhesion molecules such as fibronectin III domains (*15*), the ZAG RGD is located in a β strand rather than a loop.

The quaternary arrangement of the ZAG α3 domain with respect to the α1-α2 platform differs from that found in β_2M-binding class I and class I–related proteins. The overall shape of ZAG is similar to an inverted "L," in which the long axis of the α3 domain is roughly perpendicular to the flat side of the α1-α2 platform, whereas the comparable angle is acute in β_2M-binding proteins (Fig. 2A). In those proteins, β_2M interacts with the α3 domain and the underside of the α1-α2 platform and is typically required for stability (*16*). The displacement of the ZAG α3 domain compared to its class I counterpart results in the inability of β_2M to optimally contact the α3 and α1-α2 domains of ZAG (*17*), contributing to ZAG's lack of affinity for β_2M. The high thermal stability of ZAG in the absence of β_2M (*9*) can be explained by a network of hydrogen bonds between α3 and α1-α2 that are not present in β_2M-binding class I proteins (Fig. 2B). In addition to the extra hydrogen bonds, the loop connecting β strand 4 to the helical region of the ZAG α1 domain platform (residues 51 to 54) and the loop connecting strands D to E in the α3 domain (residues 236 to 241) are closer together than their class I counterparts, contributing to the burial of a larger interdomain surface in ZAG (970 $Å^2$ total) than in classical class I molecules (660 $Å^2$ in HLA-A2) (*11*). There is some flexibility in the position of the ZAG α3 domain relative to α1-α2, as demonstrated by different interdomain relationships of the four ZAG molecules in the crystallographic asymmetric unit (Fig. 2A), yet ZAG is not particularly protease-sensitive at the platform-

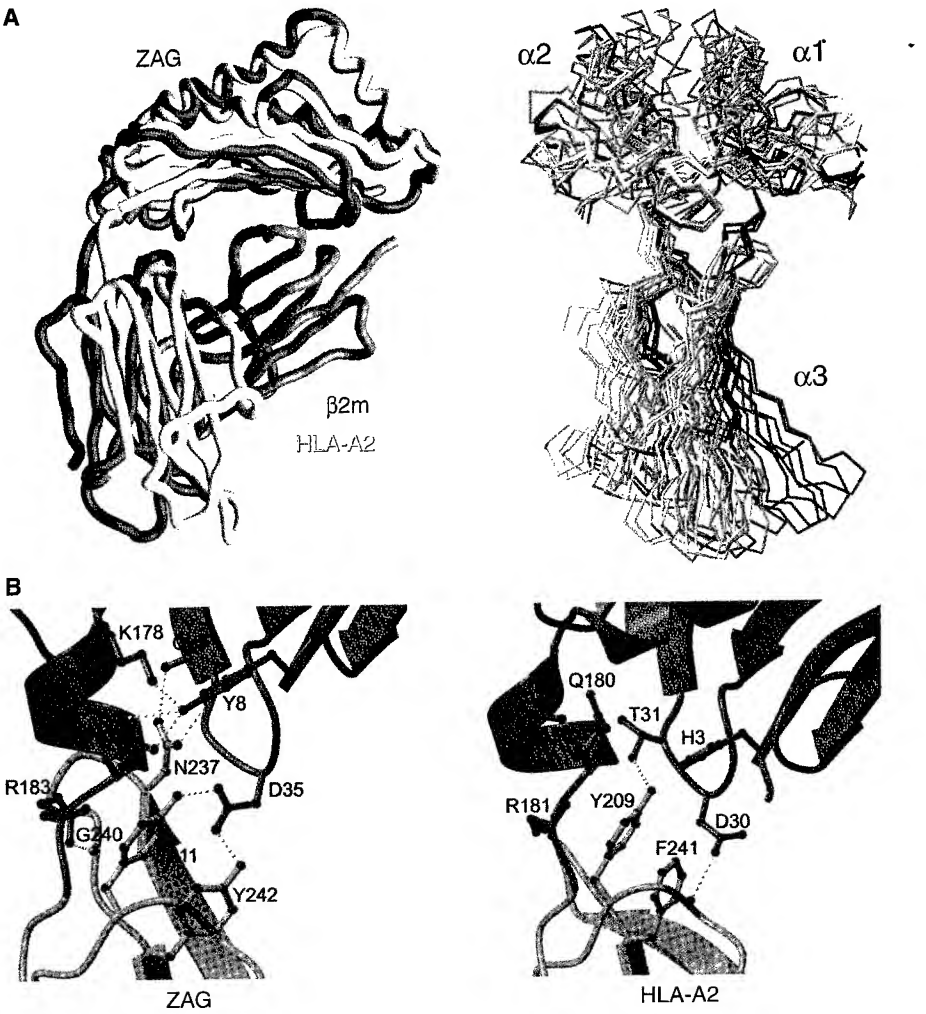

Fig. 2. Structural comparisons of ZAG, class I, and class I–related proteins. (**A**) (Left) Comparison of the structures of ZAG and HLA-A2 (*11*). (Right) Comparison of the four ZAG molecules in the crystallographic asymmetric unit (magenta) with the heavy chains of human class I MHC (yellow; PDB codes 1hhh, 1vac, 2clr) and β_2M-binding class I MHC homologs (green; FcRn, CD1, HFE) (*11*). Superpositions were based on the Cα atoms in the platform domains, using 2clr as a reference molecule. The position of the ZAG α3 domain with respect to its α1-α2 platform falls out of the range of the positions of the α3 domains of the β_2M-binding class I proteins. Differences in the platform-α3 interdomain relationships in the four ZAG molecules demonstrate that there is flexibility in the position of the ZAG α3 domain relative to α1-α2. However, the overall similarity of the four molecules, which are subjected to different crystal packing forces, rule out that ZAG's shape is an artifact of crystallization. (**B**) Close-up comparison of the interface between α1-α2 (blue) and α3 (green) in ZAG and HLA-A2 (*11*). Additional H-bonds and a larger interdomain surface area stabilize ZAG compared with class I molecules, whose heavy chains are stabilized by interactions with β_2M. Figures were made as described (*23*).

α3 hinge or any other region (*18*). Although the quaternary structure of ZAG differs significantly from the heavy chains of class I and class I–like structures, the differences are less than anticipated from the absence of the β_2M light chain. The overall similarity between ZAG and class I MHC heavy chains contrasts with the large interdomain rearrangements observed in the crystal structure of MIC-A (*10*).

Despite the similarity between ZAG and class I molecules, structural features of the ZAG α3 domain make it unlikely to associate with the T cell coreceptor CD8. Of 15 class I heavy-chain residues identified at the CD8 binding site in the HLA-A2/CD8 cocrystal structure (*19*), only one is conserved between class I and ZAG sequences (class I Asp^{122}, ZAG Asp^{123}) (*4*). It is not possible to rule out an interaction between ZAG and T cell receptors, because the class I MHC residues that contact these receptors are not particularly conserved (*20*).

Although ZAG does not associate with peptides (*9*), the helices in both the α1 and α2 domains are almost identically positioned to their counterparts in peptide-binding class I MHC molecules (Fig. 3A) (*21*). Thus, the ZAG platform includes an open groove, by contrast to the narrowed or closed grooves observed in other class I homologs that do not bind peptides

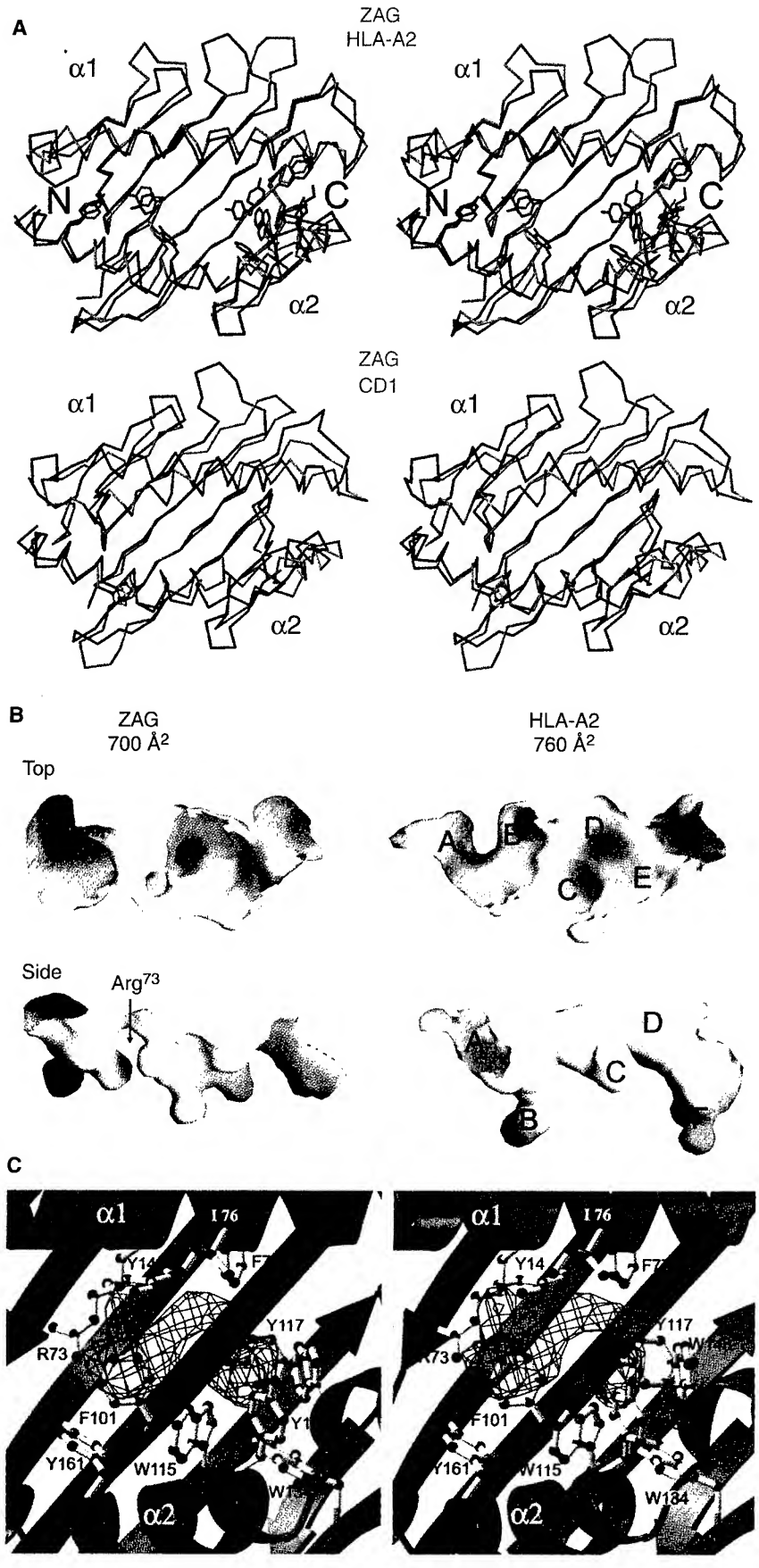

Fig. 3. Comparisons of the grooves of ZAG, class I, and class I–related proteins. (**A**) Stereoview comparisons of the α1-α2 platforms of ZAG and class I proteins that contain open antigen-binding grooves. (Top) ZAG is compared with HLA-A2, a classical class I MHC molecule. "N" and "C" indicate the orientation with respect to the NH_2- and COOH-termini of a peptide bound in the HLA-A2 groove. Side chains in common between the ZAG and HLA-A2 grooves (Table 2) are highlighted on the Cα backbones. Although many of the ZAG groove residues are chemically identical to their counterparts in HLA-A2, the conformations of the side chain, the backbone, or both, of these residues are generally different; thus, the grooves have different shapes [see (B)]. (Bottom) Comparison of ZAG and CD1. Although the ZAG and CD1 grooves both bind nonpeptidic ligands, the ZAG groove is smaller and shallower. Prolines within the α2 domain helices of ZAG (Pro^{167}) and CD1 (Pro^{169}) are highlighted. The proline in the ZAG helix is accommodated without significant distortion (*24*), as previously seen for the analogous proline within the HFE helix (Pro^{166}) (*11*). (**B**) Extruded groove pockets of ZAG and HLA-A2. Cut-away groove molecular surfaces (*11*) are shown from above (top) and the side (bottom) with electrostatic potentials (*23*), in which positive potential is blue, neutral is white, and negative potential is red. The approximate locations of pockets A through F (Table 2) are indicated on the HLA-A2 surfaces. The molecular surface of the central portion of the ZAG groove is nearly neutral except for the contribution of Arg^{73} and, like CD1 (*11*), is hydrophobic compared to the grooves of the class I molecules. Calculated groove surface areas (*11*) are indicated for ZAG and HLA-A2. For comparison, the groove surface areas for other MHC homologs are ~1440 Å² (CD1), ~235 Å² (FcRn), and ~415 Å² (HFE) (*11*). (**C**) Stereoview of electron density (from a 2.9 Å MIRAS, NCS averaged, figure-of-merit weighted electron density map contoured at 1σ) corresponding to the ZAG ligand superimposed upon a ribbon diagram of the ZAG α1-α2 platform. Residues within 4.5 Å of the density (Table 2) are highlighted in ball-and-stick representation. Figures were made as described (*23*).

[FcRn (*11*), HFE (*11*), and MIC-A (*10*)]. Despite the fact that most of the residues within the COOH-terminal portion of the ZAG α2 domain helix are chemically identical to their class I counterparts (Table 2), the ZAG groove has a different shape than a typical class I MHC peptide-binding groove (Fig. 3B). Differences in side chain conformations (Fig. 3A) and side chain substitutions create the altered shape of the ZAG groove, which can be described as containing three pockets: a large, predominantly hydrophobic central pocket and two smaller, more acidic flanking pockets (Fig. 3B). The boundary on the left of the central pocket (Fig. 3B) is created by the side chain of Arg^{73}, which points into the groove to separate the central and left pockets. Class I MHC grooves contain a smaller side chain at this position (His^{70} in HLA-A2) (*4*). Because of Arg^{73} and other side chain substitutions or conformational differences relative to class I molecules, the ZAG groove cannot accommodate even a polyalanine version of an eight- or nine-residue peptide in a class I–binding conformation (Table 2).

Instead of a peptide, the ZAG groove contains an as yet unidentified ligand that cocrystallizes with the protein. Electron density that cannot be accounted for by the amino acid sequence or N-linked carbohydrates is found in the central pocket of the ZAG groove (Figs. 1A and 3C). The density is a curved nonbranched tube, lacking the characteristic protrusions of peptides, that is situated near a cluster of hydrophobic amino acids (three tryptophans, four tyrosines, an isoleucine, and two phenylalanines) and the positively charged side chain of Arg^{73} (Table 2). Although it is not possible to unambiguously identify the compound or compounds in the ZAG groove at the current resolution of the electron density maps, previous biochemical studies rule out that the density corresponds to peptide or mixture of peptides (*9*). The composition of the ZAG groove residues near the density (Table 2) suggests that it represents one or more small hydrophobic molecules, perhaps negatively charged. Chloroform-methanol extractions and acid eluates of ZAG as well as the intact protein have been analyzed by gas chromatography, electrospray, or matrix-assisted laser desorption/ionization mass spectrometry, or a combination of these methods (*22*). The ligand has not yet been detected, but in the absence of information about the chemical nature, size, and ionic state of the ligand, these results cannot be considered conclusive. Electrospray analyses of proteolytic fragments of ZAG reveal that the ligand is not covalently bound (*22*).

Classical class I MHC molecules are extremely polymorphic, whereas ZAG exhibits species-specific variations but little or no genetic polymorphism (*4, 5*). Most of the allele-specific variations of class I MHC molecules map to residues within the peptide-binding groove that interact with peptides (Table 2) (*5*). This property of class I molecules results in allele-specific peptide-binding motifs, such that individual class I molecules show distinct preferences for binding peptides (*5*). In contrast, residues within the ZAG groove are mostly conserved, and those residues closest to the ZAG ligand are completely conserved (Table 2), even though human and rodent ZAG share only ~56% sequence identity (*4*). Taken together with ZAG's lack of polymorphism, these observations imply that human and rodent ZAG carry a single compound, or single class of compounds, related to their function in lipid catabolism.

The crystallographic analysis of ZAG reveals a structure with surprising similarity to classical class I molecules despite ZAG's inability to bind peptides or β_2M (*9*). ZAG and MHC-related proteins such as FcRn (*11*) and HFE (*11*) have adapted the same basic fold to perform widely different roles within and outside of the immune system. These molecules illustrate the versatility of the MHC fold and raise intriguing questions about the ancestral function and evolutionary relationships of MHC and MHC-related proteins.

Table 2. Comparison of residues in α1-α2 grooves of ZAG and a class I MHC molecule. Uppercase letters in any of the HLA-A2 and ZAG columns indicate residues that are conserved in human class I MHC sequences (*4*) or residues that are conserved in human, mouse, and rat ZAG (*4*). Pocket residues in the peptide-binding groove of HLA-A2 are defined as having ≥5.0 Å² accessible surface area to a 1.4 Å probe, but <5.0 Å² accessible surface area to a 5 Å probe (*11*). HLA-A2 residues marked with an asterisk were originally defined as pocket residues by M. A. Saper *et al.* (*11*) but are accessible to a 5.0 Å probe. The HLA-A2 residues designated as "NONE" (conserved) or "none" (not conserved) do not meet the criteria for being in a pocket, but are listed for comparison with ZAG. Pocket letter names (uppercase letters, conserved residues) refer to well-characterized pockets in the peptide-binding grooves of class I MHC molecules (*5, 11*). ZAG residues analogous to class I pocket residues are designated as "POCKET" (conserved) or "pocket" (not conserved) if they meet the criteria for pocket residues in HLA-A2, "BURIED" or "buried" if they have ≤5.0 Å² accessible surface area to a 1.4 Å probe and ≤5.0 Å² accessible surface area to a 5 Å probe, and "EXPOSED" or "exposed" if they have ≥5.0 Å² accessible surface area to a 1.4 Å probe and ≥5.0 Å² accessible surface area to a 5 Å probe. Surface areas were calculated (*11*) using the coordinates of HLA-A2 (excluding water molecules and bound peptide) and ZAG. Steric clashes with polyalanine peptides bound to ZAG in their class I–binding configuration were defined as described in the structural analysis of HFE (*11*).

HLA-A2	Pocket	ZAG	Pocket	Clash with peptide?	≤4.5 Å of ZAG ligand?
MET-5	A	LEU-10	BURIED	NO	NO
TYR-7	A,B	tyr-12	pocket	yes	no
phe-9	b,c	TYR-14	POCKET	NO	YES
met-45	b	ser-48	pocket	no	no
tyr-59	a	ASP-62	EXPOSED	NO	NO
glu-63	a,b	asp-66	pocket	no	no
lys-66*	a	LEU-69	EXPOSED	YES	NO
val-67	b	GLN-70	POCKET	YES	NO
his-70	b,c	ARG-73	POCKET	YES	YES
thr-73*	c	ILE-76	EXPOSED	YES	YES
his-74	none	PHE-77	POCKET	NO	YES
val-76	none	glu-79	exposed	yes	no
asp-77*	f	THR-80	POCKET	YES	NO
thr-80*	f	ASP-83	EXPOSED	YES	NO
leu-81	f	ILE-84	POCKET	NO	NO
arg-97	c,e	GLY-99	POCKET	NO	NO
tyr-99	a,b,c,d	PHE-101	POCKET	NO	YES
his-114	c,d,e	TRP-115	POCKET	NO	YES
tyr-116	c,f	TYR-117	POCKET	NO	YES
TYR-118	F	TYR-119	POCKET	NO	NO
TYR-123	F	tyr-124	pocket	no	no
ILE-124	F	ILE-125	BURIED	NO	NO
TRP-133	NONE	TRP-134	POCKET	NO	YES
thr-143	f	THR-144	POCKET	YES	NO
LYS-146	NONE	LYS-147	EXPOSED	YES	NO
trp-147	e,f	TRP-148	POCKET	NO	YES
val-152	e	TYR-154	EXPOSED	NO	YES
leu-156	d,e	ALA-158	POCKET	NO	NO
TYR-159	A,D	TYR-161	POCKET	YES	YES
trp-167	a	thr-169	pocket	yes	no
tyr-171	a	TYR-173	POCKET	YES	NO

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14. Extensive carbohydrate density is found at Asn^{239} (nine ordered carbohydrate residues in molecule 2) and to a much lesser extent at Asn^{89} and Asn^{108} in all four ZAG molecules (Fig. 1) (*13*). Crystal structures of glycoproteins rarely show more than three ordered carbohydrate residues at each glycosylation site [D. E. Vaughn and P. J. Bjorkman, *Structure* **6**, 63 (1998)]. The Asn in the fourth potential N-linked glycosylation site (Asn^{92}) does not show density corresponding to carbohydrate. The bond between Asn^{92} and Gly^{93} can be cleaved by hydroxylamine, confirming that Asn^{92} is not glycosylated (*18*).
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24. ZAG, CD1, HFE, and FcRn contain prolines within their α2 domain helices at a position corresponding to Val^{165} in classical class I MHC molecules (*4*). The FcRn and CD1 helices are kinked at a position near their proline residues, whereas the ZAG and HFE helices are similar to the α2 domain helices of class I molecules (*11*). Substitution of Val^{165} for proline in the mouse class I molecule H-2D^d did not interfere with binding and presentation of peptides to T cells, suggesting that no major structural rearrangements occurred [D. Plaksin, K. Polakova, M. G. Mage, D. H. Margulies, *J. Immunol.* **159**, 4408 (1997)].
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Caltech biologists reveal structure of protein responsible for weight loss in cancer and AIDS patients

PASADENA-Caltech biologists have determined the three-dimensional structure of a protein that causes wasting in cancer and AIDS patients. The discovery could lead to new strategies for controlling weight loss in patients with devastating illnesses-and conversely, perhaps new strategies for fighting obesity.

The protein is commonly known as ZAG and is found in most bodily fluids. But researchers have been aware for some time that the protein is particularly abundant in patients who have cancer.

More recently, researchers have discovered that the protein is involved in the wasting syndrome known as cachexia, which is associated with both cancer and AIDS.

"This protein has something to do with fat metabolism," says Pamela Bjorkman, a professor of biology at Caltech and associate investigator of the Howard Hughes Medical Institute. Bjorkman and her team recently published a paper in the journal Science showing ZAG's structure.

One of the most noteworthy features of the structure is the resemblance between ZAG and a family of proteins known as class I major histocompatibility complex molecules, or MHC.

"MHC proteins have a large groove that binds a peptide derived from a pathogen," says Bjorkman, explaining that their new picture of the ZAG crystal shows an unexpected blob in the ZAG counterpart of the MHC peptide binding grove.

Human ZAG

Three-dimensional structure of a human ZAG.

Related Links

Dr. Pamela Bjorkman

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"It's not a peptide, but some organic molecule," she says. "We suspect that it is involved in the function of ZAG. If this compound is involved in breaking down lipids, then maybe you could design a drug that replaces it and interfere with lipid breakdown."

According to Bjorkman, other research shows that tumor cells themselves seem to stimulate the body to overproduce ZAG somehow, which in turn leads to the breakdown of body fat.

Thus, people suffering from cachexia don't lose body weight because they don't eat, but because the fat in their bodies is ultimately destroyed by an interaction involving ZAG.

An intervention to stop the wasting, then, might be to disrupt the overexpression of ZAG, and this might be accomplished with monoclonal antibodies or small molecules that bind to ZAG, she says.

The research appeared in the March 19 issue of Science, and was also the subject of an article in HHMI news, published by the Howard Hughes Medical Institute.

The other authors of the paper are Luis Sanchez and Arthur Chirino, both senior research fellows in Bjorkman's lab.

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HHMI NEWS
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Structure of Fat-Depleting Protein Reveals Important Functional Clues

In the Groove
Finding a molecule that binds in ZAG's (red) groove may yield the template for a new class of obesity drugs.

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March 19, 1999—Following a mysterious crevice in a molecule that causes severe weight loss in some cancer patients may turn up a new generation of drugs to treat clinical obesity, say researchers from the Howard Hughes Medical Institute (HHMI) at the California Institute of Technology.

Zn-α_2-glycoprotein, otherwise known as ZAG, occurs naturally in most body fluids, including blood, sweat, saliva, and urine. Researchers first isolated ZAG from blood samples more than 30 years ago, "but it's been a molecule in search of a function for a long time," said Pamela Bjorkman, an HHMI investigator at the California Institute of Technology in Pasadena. Bjorkman, Luís Sánchez and HHMI associate Arthur Chirino published ZAG's structure in the March 19, 1999, issue of the journal *Science*.

One of the mysteries surrounding ZAG is that it seems to be related to a large family of proteins known as class I major histocompatibility complex (MHC) molecules. These molecules bind to small pieces of other proteins, known as antigenic peptides, and in so doing trigger an immune response to invading microorganisms and viruses. Yet despite ZAG's similarity to MHC proteins, it apparently does not have a role in the immune system, Bjorkman said.

Last year, however, researchers at Aston University in Birmingham, U.K., discovered that ZAG is involved in cachexia, a wasting syndrome that can affect people

with cancer, AIDS and other terminal illnesses. Cachexia can result in rapid, life-threatening weight loss and lead to shedding of both fat and muscle.

ZAG appears to drive the fat-loss in cancer patients. When the British investigators added ZAG to fat cells, the cells rapidly metabolized lipids, a major component of fat. Further evidence of ZAG's role in fat breakdown came when researchers, who fed the protein to genetically obese mice, noticed that he mice lost body fat even though they maintained normal eating habits. If ZAG produces the same effect in humans and is deemed safe, Bjorkman adds, it could provide a long-sought treatment for clinical obesity.

Understanding how ZAG boosts fat breakdown is a necessary precursor to any plans to develop ZAG-based obesity drugs. Knowing that structure often reveals details about function, Bjorkman's group created a molecular map of ZAG's structure using x-ray crystallography, which provides a three-dimensional picture revealing the location of each of the molecule's atoms. Surprisingly, this image showed that ZAG's structure is even more like that of its MHC relatives than researchers had previously thought.

One distinguishing feature of MHC proteins is a large groove that the molecules use to bind antigenic peptides. Bjorkman's group discovered that ZAG also has this groove, but unlike MHC molecules, ZAG's groove doesn't bind peptides. "We found that the ZAG groove contains some sort of compound. We know that it's not a peptide, but we haven't identified it yet," Bjorkman said. A search of small molecule databases for molecules capable of fitting in the groove failed to find any good candidates among known compounds.

Bjorkman's top priority is to find the molecule that binds in ZAG's groove, since this unknown partner may hold the key to understanding how ZAG promotes fat breakdown. "There's a big hint there," said Bjorkman, "we just don't know what the answer is yet. But once we identify this compound, it's possible you could inhibit the binding of this molecule to ZAG as a treatment for cachexia." She added that such a strategy might be particularly helpful for breast cancer patients since ZAG accumulates in 40 percent of cancerous breast tissue.

The information about ZAG's structure also provides important clues about the evolution of the MHC protein family. Bjorkman explained that prior to this and other structural work on MHC-related proteins, researchers believed that the MHC molecule groove evolved to bind

antigenic peptides. Given the fact that ZAG shares this structure yet is not involved in the immune system suggests that the groove may have evolved for a different reason, she says.

"Perhaps the groove first evolved for a purpose other than antigen binding and MHC molecules have just capitalized on its existence," said Bjorkman. "But there's still a lot we don't understand." Determining ZAG's structure, she added, is simply the first step towards understanding how the molecule does its job.

illustration: Pamela Bjorkman

Top of Page...

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